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http://dx.doi.org/10.1289/ehp.1205823

Online 19 March 2013

NIEHS
National Institute of Environmental Health Sciences

National Institutes of Health
U.S. Department of Health and Human Services

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Running Head: BPA disrupts estrogen synthesis in the ovary

Key words: 17β-estradiol, aromatase, bisphenol A, follicular atresia, luteal regression, ovary, steroidogenic acute regulatory protein

Grant support: This work was supported by the National Research Foundation of Korea(NRF) grant funded by the Korean Government(MEST) (2009-0093191).

None of the authors has any actual or potential competing financial interests to declare.

Abbreviations

17β-HSD, 17 beta-hydroxysteroid dehydrogenase

3β-HSD, 3 beta-hydroxysteroid dehydrogenase

ANOVA, analysis of variance

BPA, bisphenol A

BW, body weight

CL, corpus luteum

CVs, coefficients of variation

CYP17A1, cytochrome P450 17A1

E2, 17β-estradiol

EB, estrogen benzoate

ELISA, enzyme-linked immunosorbent assay; H_2O_2, hydrogen peroxide

EPA, the U.S. environmental protection agency

FSH, follicle stimulating hormone

GTH, gonadotropin

HEPES, hydroxyethyl piperazineethanesulfonic acid

LH, luteinizing hormone

LOAEL, the lowest observed adverse effect level

P450arom, cytochrome P450 aromatase

P450scc, cytochrome P450 side chain cleavage

PBS, phosphate buffered saline

PCNA, proliferating cell nuclear antigen

PCOS, polycystic ovarian syndrome

StAR, steroidogenic acute regulatory protein

T, testosterone

TBS-T, tris-buffered saline with tween-20

TBS, tris-buffered saline

Abstract

BACKGROUND: Bisphenol A (BPA) has been detected in human body fluids, such as serum and ovarian follicular fluids. Several reports indicate that BPA exposure is associated with the occurrence of several female reproductive diseases due to the disruption of steroid hormone biosynthesis in the adult ovary.

OBJECTIVE: We hypothesized that long-term exposure to low concentrations of BPA disrupts 17β-estradiol (E2) production in granulosa cells via an alteration of steroidogenic proteins in ovarian cells.

METHODS: Adult female rats received BPA for 90 days by daily gavage at doses of 0, 0.001, or 0.1 mg/kg body weight (BW). Serum levels of E2, testosterone (T), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) were determined. Furthermore, the expressions of steroidogenic acute regulatory protein (StAR), P450 side-chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase isomerase (3β-HSD), and aromatase cytochrome P450 (P450arom) were analyzed in the ovary.

RESULTS: Exposure to BPA significantly decreased E2 serum concentration, which was accompanied by augmented follicular atresia and luteal regression via increase of caspase-3-associated apoptosis in ovarian cells. Following BPA exposure, P450arom and StAR protein expression levels significantly decreased in granulosa and theca-interstitial (T-I) cells,

respectively. However, P450scc and 3β-HSD protein levels remained unchanged. The increase in LH levels appeared to be associated with the decreased synthesis of T in T-I cells after BPA exposure via homeostatic positive feedback regulation.

CONCLUSIONS: BPA exposure during adulthood can disturb the maintenance of normal ovarian functions by reducing E2. The steroidogenic proteins StAR and P450arom appear to be targeted by BPA.

Introduction

Bisphenol A [BPA; 2,2-bis-(4-hydroxyphenyl)propane] is a plasticizer that is widely used to produce polycarbonate plastic, epoxy resin, and unsaturated polystyrene. BPA can be leached from the linings of food cans, polycarbonate baby bottles and other beverage containers, dental sealants and composites, polyvinylchloride plastics, and recycled thermal paper, resulting in human exposure to BPA (Vandenberg et al. 2007). In fact, BPA has been detected in human serum (Takeuchi and Tsutsumi 2002), urine (Calafat et al. 2008), breast milk (Ye et al. 2006), and ovarian follicular fluids (Ikezuki et al. 2002). The increased incidence of BPA exposure in humans is suspected to be associated with the occurrence of various reproductive diseases and health outcomes. These include male sexual dysfunction (Li et al. 2010), recurrent miscarriage (Sugiura-Ogasawara et al. 2005), premature delivery (Cantonwine et al. 2010), and polycystic ovarian syndrome (PCOS) (Kandaraki et al. 2011).

In animal experiments, BPA exposure has been shown to have adverse effects on the reproductive system (Hunt et al. 2009). In female reproduction, neonatal or perinatal exposure to BPA caused significant histological changes in the reproductive tract (Newbold et al. 2007; Newbold et al. 2009), alteration of estrous cyclicity (Rubin et al. 2001), decreased reproductive capacity (Cabaton et al. 2011), and changes in hormonal levels (Fernández et al. 2009; Rubin et

al. 2001) later in adult life. Particularly in the ovary, disruption of follicular development (Adewale et al. 2009), reduction of the pool of primordial follicles (Rodríguez et al. 2010), and the occurrence of PCOS-like structures (Fernández et al. 2010) have been observed after BPA exposure during the neonatal period. However, the effect of BPA exposure on the alteration of ovarian steroidogenesis in adult animals is yet to be elucidated. It has been shown that BPA treatment alters steroid hormone production in granulosa cells (Mlynarcíková et al. 2005; Zhou et al. 2008; Grasselli et al. 2010). Furthermore, the effect of BPA on steroidogenesis has been demonstrated recently in a mouse follicle culture system (Peretz et al. 2011). Nevertheless, the precise cellular and biochemical mechanism(s) by which BPA affects ovarian steroidogenesis have not yet been identified in animals that are chronically exposed to BPA during adulthood.

We hypothesized that the adult ovary is susceptible to BPA *in vivo* and that long-term exposure to low concentrations of BPA disrupts 17β-estradiol (E2) production by granulosa cells via the alteration of steroidogenic proteins in ovarian cells, such as steroidogenic acute regulatory protein (StAR), P450 side-chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase isomerase (3β-HSD), 17α-hydroxy/C17–20lase (P450c17; CYP17A1), and aromatase cytochrome P450 (P450arom). To test this hypothesis, we examined the expression levels of

these proteins in relationship to serum E2 levels and evaluated cellular and histological alterations in the ovary.

Materials and Methods

Materials

Monoclonal Anti-actin (mouse IgG2a isotype), anti-b-tubulin, bisphenol A, Bouin's solution, corn oil, dimethyl sulfoxide (DMSO), hematoxylin, HEPES, medium 199, tryphan blue, and Tween-20 were purchased from Sigma (St Louis, MO, USA). Anti-aromatase was purchased from Acris Antibodies (San Diego, CA, USA). Anti-calbindin-D9k was obtained from Swant Swiss Antibodies (Bellinzona, Switzerland). Antibody specific for cleaved (active form) caspase-3 antibody was purchased from Cell Signaling (Beverly, MA, USA). Anti-FSH antibody was obtained from AbD Serotec (Kidlington, UK). Anti-3β-HSD, anti-CYP17A1, PCNA, and rabbit IgG antibodies were purchased from Santa Cruz Biotech (Delaware, CA, USA). Anti-P450scc antibody was obtained from Chemicon (Temecula, CA, USA). Anti-StAR and anti-LH antibodies were obtained from Abcam (Cambridge, UK).

Animals and BPA exposure

Adult female Sprague-Dawley rats (8–week-old, 200–250 g) were purchased from SamTako Bio-Korea (Osan, Korea). The rats were housed in a climate-controlled (21 ± 2°C) animal room at a constant 12-h light and 12-h dark cycle, with unlimited access to rat chow. All procedures were performed in accordance with protocols approved by the Dong-A University Animal Care and Use Committee. The animals were treated humanely and with regard for alleviation of suffering. The rats received BPA for 90 days by daily gavage at doses of 0.001 (low dose) or 0.1 (high dose) mg/kg body weight (BW) (n = 30 rats per dose). The estrogenic control rat group (n = 30) received estradiol benzoate (EB; 0.001 mg/kg BW) instead of BPA. Control animals (n = 30) received the same weight-based volume of vehicle (0.5% DMSO in corn oil). The lowest observed adverse effect level (LOAEL) for BPA established by the U.S. Environmental Protection Agency (EPA) is 50 mg/kg BW per day and the EPA reference dose (and U.S. Food and Drug Administration acceptable daily intake) is 50 μg/kg BW per day (U.S. EPA 1993). After 90 days of daily gavage, a portion of the rats (n = 18 per dose) were sacrificed consistently on the day of normal estrus phase [routinely identifiable by the presence of large numbers (at least about 50%) of needle-like cornified (or keratinized) cells] by carbon dioxide asphyxiation and the right ovaries and uterine horns were removed and fixed in Bouin's fixative for histological examination. The left ovaries were placed in cold phosphate buffer solution (PBS)

for collection of granulosa cells and the left uterine horns were snap frozen for further biochemical analysis. The rest of the rats (n = 12 per dose) were continuously examined for the determination of estrus cycle staging.

Hormone assays

E2 and T serum concentrations were determined in duplicate samples using E2 and T enzyme-linked immunosorbent assay (ELISA) kits (IBL, Hamburg, Germany) according to manufacturer's instructions. The sensitivity of the E2 assay was 9.71 pg/ml and the intra- and inter-assay coefficients of variation (CVs) were 2.7% and 7.2%, respectively. The sensitivity of the T assay was 0.08 ng/ml and the intra- and inter-assay CVs were 3.3% and 6.7%, respectively. Serum concentrations of follicle stimulating hormone (FSH) and luteinizing hormone (LH) were determined for duplicate samples using FSH (ELIZEN Rat FSH, ZenTech, Angleur, Belgium) and LH (LH DETECT®, INRA, Nouzilly, France) ELISA kits. The sensitivity of the FSH assay was 0.2 ng/ml and the intra- and inter-assay CVs were 4.7% and 8.4%, respectively. The sensitivity of the LH assay was 0.01 ng/ml and the intra- and inter-assay CVs were 4.2% and 8.1%, respectively.

Estrus cycle staging

Vaginal smears were prepared from each animal daily between 8:00 am and 10:00 am by lavage with 0.9% saline and the fluid was spotted thinly on a microscope slide. The dried slides were stained with 0.1% trypan blue in deionized water and were allowed to dry. The determination of the estrus cycle stage was based on microscopic examination, as described (Westwood, 2008). Vaginal cytology was examined for 45 consecutive days starting day after completion of BPA gavage. However, the test was not performed from day 15 to day 30 to avoid mechanical stress on the vaginas of the rats. An altered proportion of the estrus phase was calculated by dividing the number of estrus days by the total number of days that the estrus cycle was tested for (30 days).

Granulosa cell isolation and collection of residual ovaries

Granulosa cells were collected by follicular puncture as described previously (Rao et al. 1991). Briefly, granulosa cells from ovaries were harvested in ice-cold M199 medium supplemented with hydroxyethyl piperazineethanesulfonic acid (HEPES) (25 mM; pH 7.4) by follicle puncture with a 27-gauge hypodermic needle and centrifuged at 900× *g* for 5 min. The supernatant was discarded, and the pellet was immediately frozen on dry ice and stored at -80°C. The residual

ovaries (retaining theca-interstitial cells [T-I]) were thoroughly washed with M199 to release undissociated granulosa cells, transferred into clean tubes, frozen on dry ice, and stored at -80°C.

Immunohistochemistry and histochemical staining for collagen fibers

For immunohistochemical staining, deparaffinized and hydrated ovary and uterus sections were treated in 3% H_2O_2 for 5 min and rinsed with PBS for 15 min. Subsequently, the Vectastain ABC kit (Vector Lab., Burlingame, CA, USA) was used, according to manufacturer's instructions. The nuclei were counterstained with hematoxylin. For negative controls, rabbit IgG (1 mg/ml) instead of the primary antibodies was added to the reaction. In the ovarian sections, the number of atretic follicles and regressing corpus luteum that retained at least 1 caspase-3-positive cell was counted and divided by the total number of follicles and corpus luteum, respectively, in order to calculate the incidence (%) of follicular atresia and luteal regression. Histochemical staining for collagen fibers in uterine tissues was performed using the ACCUSTAIN Trichrome Stains (Gomory method) kit (Sigma, St Louis, MO, USA) according to manufacturer's instructions.

Western blot analysis

Western blot analysis was performed as describe previously (Chung et al. 2011). Briefly, granulosa cell were lysed and protein samples (~30 μg) were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto a nitrocellulose membrane,

probed with a primary antibody, and labeled with horseradish peroxidase-labeled secondary antibodies. The signals detected with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Statistical analysis

Data are expressed as the mean standard deviation (± SD) of 3 or 4 separate experiments. When appropriate, data were analyzed using analysis of variance (ANOVA) test followed by Duncan's post hoc test. The means were considered significantly different at $P < 0.05$.

Results

Effect of BPA on E2 serum concentration and uterine alteration in the duration of estrus phase

To determine whether BPA exposure induces alterations of the female reproductive system, adult female Sprague-Dawley rats received a daily dose of either 0.001 (low dose) or 0.1 (high dose) mg/kg BW of BPA for 90 days by gavage. Both the low and high doses of BPA significantly decreased serum E2 concentration compared to that in control rats (Figure 1A). In contrast, the duration of the estrus phase was extended by BPA exposure (Figure 1B). Exposure to EB resulted in the same alteration patterns as observed with BPA (Figure 1A,B). All of the animals

tested for 45 days in this study continued to cycle, and most of those exposed to BPA or EB showed an extended estrus phase of 2–7 days. However, no animals in the current study entered a persistent estrus phase.

Reduced E2 levels after BPA were confirmed by alterations in uterine cell proliferation and integrity

To confirm whether the reduced E2 levels after BPA resulted in uterine tissue alterations, we examined estrogen-reactive proteins related to cell proliferation (proliferating cell nuclear antigen [PCNA]) and tissue integrity (calbindin-D9k and collagens) in the uterus. PCNA proteins were predominantly localized in the nuclei of luminal endometrial cells (Supplemental Material, Figure S1A, a–d), and the number of PCNA-positive cells was much higher in the control group (Supplemental Material, Figure S1A a) than in either the BPA- or EB-treated group (Supplemental Material, Figure S1A, b–d). The immunoreactivity for calbindin-D9k, an estrogen-responsive protein (Krisinger et al. 1992), in myometrial tissues was much more intense in the control group (Supplemental Material, Figure S1A e) than in either the BPA or EB-treated group (Supplemental Material, Figure S1A, f–h). Downregulation of PCNA and calbindin-D9k protein levels after BPA exposure were confirmed by western blot analysis (Supplemental Material, Figure S1B,C). Furthermore, trichrome staining showed that BPA exposure reduced

the amount of collagen fibers in the myometrium and endometrium of uterine tissues (Supplemental Material, Figure S2).

Increased ovarian cell apoptosis after BPA exposure correlated with augmentation of follicular atresia and luteal regression

In order to examine whether reduced E2 levels are associated with increased degenerative processes (i.e., ovarian follicular atresia and luteal regression) in the ovary, we investigated caspase-3-dependent apoptotic cell death in the follicles and corpus luteum (CL). Western blot analysis for caspase-3 in whole ovarian tissues showed that caspase-3 activation was significantly increased by BPA exposure (Figure 2A) in a dose-dependent manner (Figure 2B). Similarly, caspase-3 immunoreactivity was more frequently seen in the granulosa cells of the degenerating (atretic) follicles (Figure 2C, b–c) and in the luteal cells of the CL (Figure 2C, f–g) in the ovaries of the BPA-exposed groups compared to the controls (Figure 2C, a–e). A significantly higher number of follicles and CL retained caspase-3-positive cells in the BPA-treated ovaries compared to the controls (Figure 2D,E; Supplemental Material, Figure S3).

BPA exposure caused downregulation of P450arom protein expression in the granulosa cells of the ovarian follicles

Ovarian aromatase expressed in granulosa cells facilitates the conversion of E2 from androgens produced in the theca cells of the antral follicles. Thus, we investigated whether a change in aromatase expression was associated with E2 synthesis after BPA. Decreased levels of P450arom protein expression were evident in the granulosa cells of the BPA-exposed groups (Figure 3A,B). P450arom was predominantly localized in the granulosa cell layers of the large antral (preovulatory) follicles (Figure 3C). P450arom immunoreactivity was remarkably reduced in the BPA- and EB-exposed groups (Figure 3C, b–d and f–h) compared to those of the controls (Figure 3C, a–e).

Changes in StAR, P450scc, 3β-HSD, and CYP17A1 expression in theca-interstitial cells after BPA exposure

The E2 synthesis capability of granulosa cells is linked to the steroidogenic activity of T-I cells in terms of substrate (androgen) production and supply. Therefore, it is important to investigate the status of the major steroidogenic proteins that are involved in androgen production in these cells. Residual ovaries were analyzed by western blot analysis to monitor the changes in the expression levels of these proteins. BPA exposure resulted in a significant downregulation of

StAR expression, but P450scc and 3β-HSD levels were apparently unaffected (Figure 3D,E). CYP17A1 expression also remained unchanged after BPA exposure (Supplemental Material, Figure S4). These observations were confirmed by immunohistochemistry (Figure 3G and Supplemental Material, Figure S4C). The StAR protein was obviously decreased in the theca cells of either the BPA- or EB-exposed group (Figure 3G, b–c). In contrast, P450scc (Figure 3G, f–g), 3β-HSD (Figure 3G, j and h), and CYP17A1 (Supplemental Material, Figure S4C, b-c) protein immunoreactivities in the BPA- and EB-treated groups were similar to the controls (Figure 3G, e and Supplemental Material, Figure S4C, a, respectively). Finally, serum T levels were determined to monitor whether substrate production of E2 is altered by the decreased StAR protein content within T-I cells. The serum T concentration was decreased in the BPA-exposed groups (Figure 3F).

Effect of chronic BPA exposure on FSH and LH synthesis and release from the pituitary gland

The potential decrease in gonadotropin (FSH and LH) production caused by BPA exposure may result in decreased E2 synthesis in the ovary. To evaluate this possibility, serum FSH and LH concentrations were measured and FSH and LH protein expression levels were monitored in the pituitary glands. BPA exposure significantly increased serum LH levels (Figure 4B) and LH

protein content in the pituitary gland (Figure 4C,D). LH immunostaining revealed that the LH-positive cells were localized in the pituitary gland and their immunoreactivities were more intensein the BPA-exposed groups (Figure 4E, f–g) compared in the control group (Figure 4E-e). Serum LH levels appeared to be higher in the EB-exposed groups compared to the controls (Figure 4B), but the differences were not significant. Pituitary LH protein contents and immunoreactivity in the EB-exposed groups did not differ from those in the controls (Figure 4C,D,E-h). In contrast to LH, serum FSH levels (Figure 4A), pituitary FSH protein content (Figure 4C,D), and the immunoreactivity of FSH-positive cells in the pituitary (Figure 4E, b–c) were not altered.

Short-term exposure to BPA resulted in decreased E2 via downregulation of P450arom without gonadotropin level alteration

We performed short-term exposure experiments in adult female rats to determine whether BPA-induced E2 decreases are initially provoked by reduced pituitary gonadotropin (GTH) or by follicle loss via granulosa cell apoptosis in the earlier time points during BPA exposure. The rats received BPA for 7 or 14 days (1 or 2 weeks; wk) with the same scheme employed in the present study. Both 1 and 2 wk after BPA exposure, serum E2 concentrations were significantly decreased in the high-dose BPA- and EB-treated groups compared to those in control rats

(Supplemental Material, Figure S5). Although P450arom proteins were significantly downregulated in granulosa cells after exposure to BPA, levels of StAR, P450scc, and 3β-HSD remained unchanged in residual ovaries (Supplemental Material, Figure S6). Meanwhile, levels of serum LH and FSH including their protein contents in the pituitary were not noticeably different between the control and BPA-exposed groups (Supplemental Material, Figure S7). During treatment, apoptotic cell death (evaluated by caspase-3 activation) was not detected in the granulosa cells of any of the groups (Supplemental Material, Figure S6).

Discussion

Recent studies have suggested that BPA exposure is associated with several obstetrical and gynecological problems in humans (Cantonwine et al. 2010; Kandaraki et al. 2011; Sugiura-Ogasawara et al. 2005). Given the estrogenicity of BPA, it could presumably disturb or mimic estrogen action, which is necessary for the normal maintenance of female reproduction and hormonal balance during adulthood. To date, many animal studies have focused on perinatal or neonatal BPA exposure (Adewale et al. 2009; Cabaton et al. 2011; Fernández et al. 2010; Rodríguez et al. 2010) because these periods are prone to exogenous chemicals sensitivity. Although a considerable number of adult females are exposed to BPA worldwide, the reproductive health risks and complications of BPA exposure during adulthood have not been

precisely evaluated at the animal level. Thus, this study aimed to investigate whether BPA exposure during adulthood could affect ovarian steroidogenesis and subsequently provoke pathophysiological changes in the ovary.

The selection of environmentally relevant BPA doses is one of the most important factors for appropriate risk assessment of an exposure study. Generally, "low dose" is used to refer to environmentally relevant doses, i.e., doses resulting in serum levels close to those observed in human serum. The doses employed in the present study were 1 μg/kg BW (low dose) and 100 μg/kg BW (high dose). The low dose was 50 times lower and the high dose was 2 times higher than the EPA reference dose. We believe that the dose range selected in this study was adequate for the evaluation of actual environmental exposure effects of BPA on alterations in hormones and female reproduction *in vivo*. Furthermore, this dose range was similar to those used in previous studies (Newbold et al. 2007; Newbold et al. 2009; Cabaton et al. 2011).

In the present study, long-term BPA exposure in female adult rats caused a significant decrease in E2 serum concentration, which was accompanied by increased estrus phase duration, increased ovarian cell apoptosis, and decreased E2-regulated protein expressions and collagen content in the uterus. The E2 concentration decreases during the rat estrus cycle and is maintained at a relatively lower level during the estrus phase than in the other phases (i.e.,

diestrus and proestrus) (Kalra and Kalra 1974). Estrogen has been shown to suppress apoptosis in granulosa cells (Billig et al. 1993) and luteal cells (Goodman et al. 1998). Furthermore, it was clearly demonstrated that E2 maintains corpus luteum function (Kahn et al. 1987). Up-regulation of PCNA (Rumpel et al. 1995) and calbindin-D9k (Krisinger et al. 1992) expression levels and increased collagen content (Smith and Allison, 1966) in uterine tissues are closely correlated with increased E2 levels. These findings taken together with our results indicate that serum E2 levels were indeed reduced by BPA exposure. In particular, caspase-3-positive apoptotic cells were significantly increased in the granulosa cells of the antral follicles and in the luteal cells of the corpus luteum of rat ovaries exposed to BPA in this study, suggesting that long-term BPA exposure during adulthood causes augmentation of follicular atresia and luteal regression in the ovary. Caspase-3 has been implicated in ovarian follicular atresia and luteal regression in the rat ovary (Boone and Tsang 1998). Although ovarian follicular atresia and luteal regression are normal physiological processes for the adequate maintenance of ovarian functions, the aberrant increase in these processes can cause disturbances in follicle selection and the life span of the corpus luteum. Several *in vitro* studies support our findings: BPA treatment resulted in decreased E2 production in FSH-stimulated porcine (Mlynarcíková et al. 2005), human granulosa cells (Kwintkiewicz et al. 2010), and cultured mouse antral follicles (Peretz et al. 2011). Therefore, it

is believed that BPA has a direct adverse effect on the regulation of E2 production in granulosa cells.

Estrogen biosynthesis is catalyzed by P450arom (the product of the *CYP19 gene*). In the ovary, P450arom expression occurs predominantly in the granulosa cells of the preovulatory follicles (Stocco, 2008). Recent *in vitro* studies showed that BPA causes downregulation of P450arom mRNA expression in rat granulosa cells (Zhou et al. 2008), FSH-treated human (Kwintkiewicz et al. 2010) and rat (Quignot et al. 2012) granulosa cells, and mouse antral follicles (Peretz et al. 2011). Consistent with these results, we have demonstrated that long-term BPA exposure results in the remarkable downregulation of P450arom protein expression in the granulosa cells of the preovulatory follicles in rats. Notably, low and high doses of BPA downregulated P450arom to the same degree, indicating that BPA doses that are lower than the EPA reference dose reduce E2 levels and cause subsequent changes in the female reproductive system in adult rats. Although the precise cellular and biochemical mechanism(s) underlying P450arom protein downregulation in response to BPA exposure are presently unknown, BPA presumably has a direct negative action on the transcriptional and/or translational regulation of P450arom expression in granulosa cells. Since pituitary FSH levels were unchanged in the current study,

the influential effect of pituitary FSH can be excluded as a possible regulatory mechanism for P450arom expression.

The production of androgens in T-I cells by the activation of crucial steroidogenic proteins (StAR, P450acc, and 3β-HSD) is indispensible for estrogen synthesis in the ovary (Richards 1980). In response to pituitary LH, particularly StAR transfers cholesterol from the outer membrane to the inner mitochondrial membrane (Stocco, 2001), where the P450scc enzyme is located. In the present study, BPA exposure decreased StAR expression in T-I cells, but P450scc and 3β-HSD protein levels remained virtually unaffected. Previous studies showed that BPA treatment increases StAR and P450scc mRNA expression in cultured T-I cells (Zhou et al. 2008) but decreases StAR, P450scc, and 3β-HSD in antral follicle cultures (Peretz et al. 2011). This discrepancy could originate from differences in BPA doses, treatment duration, the *in vitro* culture system, animal species, or the levels of cellular purity and differentiation. Given that serum T levels were decreased by BPA exposure in the current study, it can be presumed that StAR is a major target protein that is affected by BPA during androgen synthesis in T-I cells.

Decreased serum levels of T and E2 ultimately stimulate the hypothalamus-pituitary gland to synthesize and release LH and FSH, respectively. Thus, the increased LH levels seen in this study were likely associated with the decreased synthesis of T in T-I cells after BPA exposure

via homeostatic reduced negative feedback regulation. Interestingly, decreased E2 levels after BPA exposure did not induce a significant increase of FSH release from the pituitary. The interpretation of this result is difficult with the information that is currently available. However, it is tempting to speculate that the prolonged status of decreased E2 synthesis due to a lack of the substrate (T) evoked the desensitization of either hypothalamic gonadotropin-releasing hormone neurons or FSH-synthesizing gonadotrophs in the pituitary. The effect of EB exposure was compared to BPA throughout this study, and the results of EB treatment were similar to the patterns in the BPA-exposed groups, but were much less pronounced. In particular, EB exposure did not affect LH production in the pituitary gland. This suggests that BPA exerts a unique cytotoxic action/mechanism(s) in addition to estrogenicity. It is currently unknown whether BPA affects steroidogenesis in ovarian cells through an estrogen receptor (ER)-mediated pathway or by direct inhibitory effect on steroidogenic proteins. The possible involvement of peroxisome proliferator-activated receptor-γ (Kwintkiewicz et al. 2010) in the regulation of steroidogenesis should be further considered in regard to BPA action in the ovary.

Finally, a potential adverse effect of BPA on E2 production in granulosa cells was constantly seen in this study, even in the short-term BPA exposure experiments, confirming the hypothesis that BPA first decreases E2 levels by directly disturbing P450arom protein expression in the

granulosa cells that are dispensable for GTH action. The low incidence of granulosa cell apoptosis after BPA exposure during these periods eliminates the possibility that follicle loss by caspase-3-mediated apoptosis is responsible for the reductions in E2 and the associated steroidogenic proteins. Taken together, we believe that the exposure of adult female rats to low concentrations of BPA initially reduces E2 synthesis by directly disrupting steroidogenesis within the ovary and that the prolonged status of reduced E2 levels subsequently provokes feedback regulation of LH increases as well as ovarian cell apoptosis. This new information will be a useful addition to the knowledge regarding the effects of BPA on female reproduction. Further studies on mechanism(s) by which BPA exhibits its adverse effects in ovarian cells at the molecular and biochemical levels are needed in the near future.

CONCLUSION

Our data indicates that long-term exposure to environmentally relevant concentrations of BPA in female adult rats results in significant reduction of serum E2 levels and an increase in ovarian cell apoptosis, which correlates to augmentation of follicular atresia and luteal regression in the ovary. The downregulation of StAR and P450arom proteins in the ovary might be a crucial step by which E2 production is interrupted after BPA exposure. Therefore, this study suggests that BPA exposure during adulthood disturbs the maintenance of normal ovarian functions by

reducing E2 and that StAR and P450arom are the definitive steroidogenic proteins that are targeted by BPA.

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Figure legends

Figure 1. Effect of BPA exposure on E2 serum concentration (A) and altered duration of the estrus phase (B). Adult female rats received 0, 0.001, or 0.1 mg/kg BW per day of BPA or 0.001 mg/kg BW per day of EB for 90 days by gavage. E2 levels were measured by ELISA (A). The estrus phase during the estrus cycle was determined by a vaginal smear (B). Values represent the mean ± SD (n = 12). *, P <0.05 and **, P <0.01 compared with each control (0 mg/kg BW).

Figure 2. Increased caspase-3-associated apoptotic cell death in ovarian cells and follicular atresia augmentation and luteal regression following BPA exposure. A: Caspase-3-associated apoptotic cell death in the ovaries was evaluated by western blot analysis using an active form-specific caspase-3 antibody. **B:** Densitometric quantification of activated caspase-3 protein levels in total ovarian protein extracts. At least 3 independent experiments were performed and the data represent the mean ± SD. *, P < 0.05 and **, P < 0.01 compared with each control (0 mg/kg BW). **C:** Immunolocalization of active caspase-3 in ovaries. AF, antral follicle; CL, corpus luteum. *Arrowheads* and a*rrows* point to active caspase-3-positive granulosa cells and luteal cells, respectively. Original magnification: 200×; bar = 60 μm. **D and E:** Changes in the proportion of atretic follicles and the regressing corpus luteum in the ovary,

respectively. Values represent the mean ± SD. *, $P < 0.05$ and **, $P < 0.01$ compared with each control (0 mg/kg BW).

Figure 3. Down-regulation of P450arom in granulosa cells and alterations in StAR, P450scc, and 3β-HSD in the T-I cells of ovarian follicles after BPA exposure. A: Western blot analysis for P450arom. **B:** Densitometric quantification of the P450arom protein level in isolated granulosa cell protein extracts. At least 3 independent experiments were performed and the data represent mean ± SD. *, $P < 0.05$ and **, $P < 0.01$ compared with each control (0 mg/kg BW). **C:** Immunohistochemical localization of P450arom in the granulosa cell layers of the large antral follicles. Microphotographs of *e–h* are enlargements of the regions marked with dotted rectangular lines in *a–d*. Original magnification: a–d, 100×; e–h, 400×; bars = 80 μm (*a-d*), 30 μm (*e-h*). **D:** Western blot analysis for StAR, P450scc, and 3β-HSD proteins. **E:** Densitometric quantification of StAR, P450scc, and 3β-HSD protein levels in residual ovaries. At least 3 independent experiments were performed and the data shown represents the mean ± SD. **, $P < 0.01$ compared with each control (0 mg/kg BW). **F:** Serum T levels measured by ELISA. Values represent the mean ± SD (n = 12). *, $P < 0.05$ and **, $P < 0.01$ compared with each control (0 mg/kg BW). AM denotes normal adult male serum. **G:** Immunohistochemical

localizations of StAR, P450scc, and 3β-HSD in T-I layers. Original magnification: 400×; bar = 30 μm.

Figure 4. Effects of BPA exposure on serum FSH and LH levels and FSH and LH protein expression in the pituitary glands. FSH **(A)** and LH **(B)** levels were measured by ELISA. Values represent the mean ± SD (n = 12). **, $P < 0.01$ in comparison with the control. **C:** Western blot analysis of FSH and LH in pituitary gland protein extracts. **D:** Densitometric quantification of FSH and LH protein levels. At least 3 independent experiments were performed and the data shown represents the mean ± SD. *, $P < 0.05$ compared with each control (0 mg/kg BW). **E:** Immunohistochemical localizations of FSH and LH in the anterior pituitary glands of rats exposed to BPA. Original magnification: 400×; bar = 30 μm.

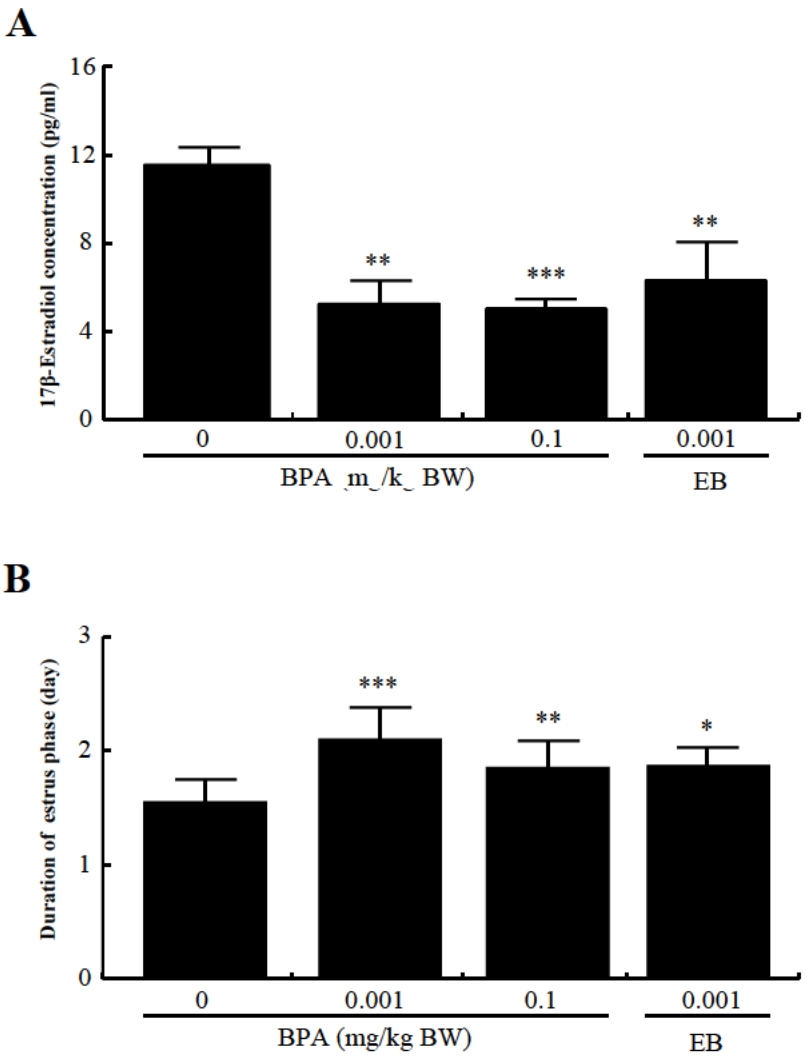
A
17β-Estradiol concentration (pg/ml)
16
12
8
4
0
**
***
**
0
0.001
0.1
0.001
BPA (mg/kg BW)
EB
B
Duration of estrus phase (day)
3
2
1
0
***
**
*
0
0.001
0.1
0.001
BPA (mg/kg BW)
EB

Figure 1

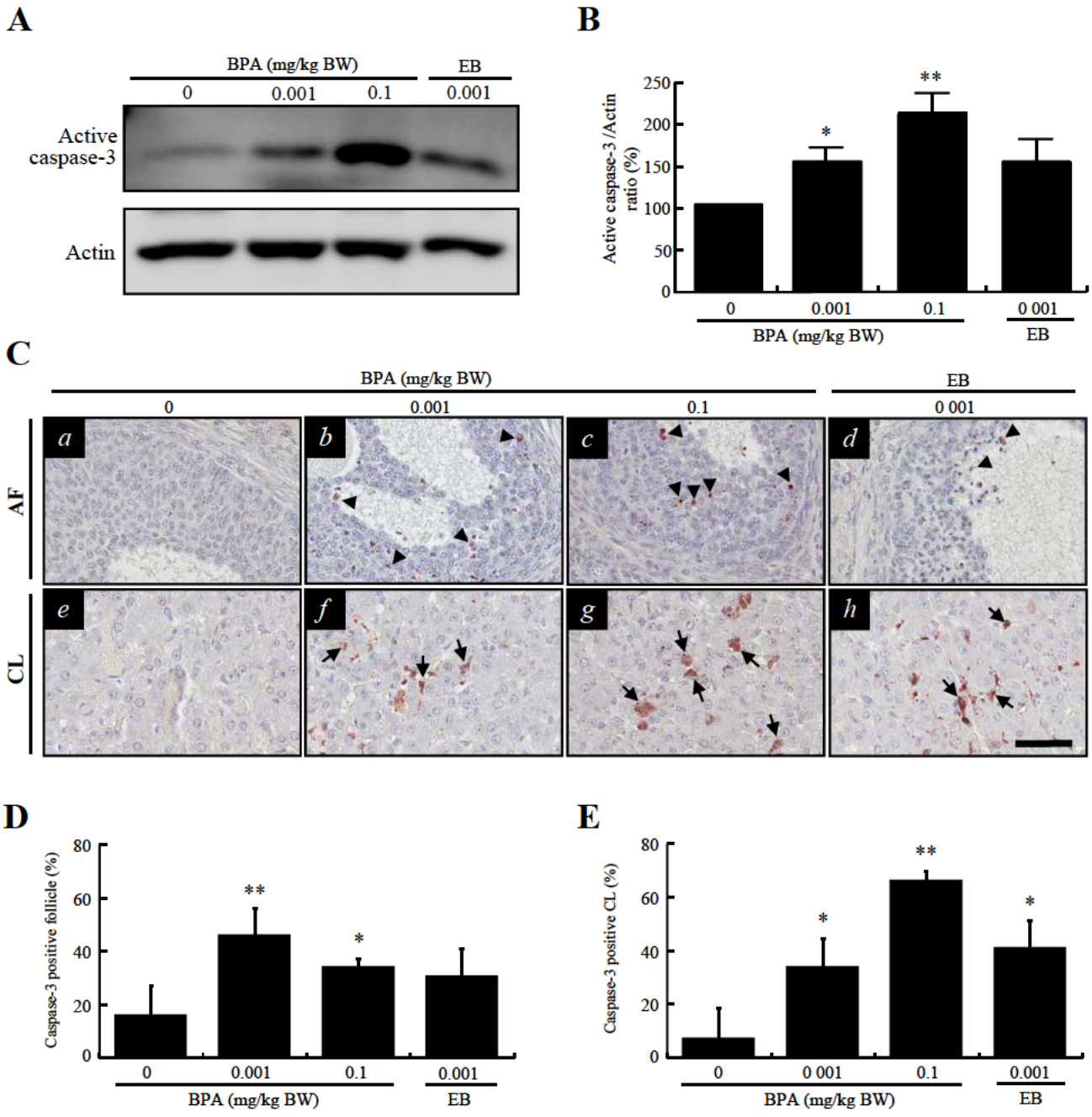
A
BPA (mg/kg BW)
EB
0
0.001
0.1
0.001
Active caspase-3
Actin
B
Active caspase-3 /Actin ratio (%)
250
200
150
100
50
0
*
**
0
0.001
0.1
0 001
BPA (mg/kg BW)
EB
C
BPA (mg/kg BW)
EB
0
0.001
0.1
0 001
AF
CL
a
b
c
d
e
f
g
h
D
Caspase-3 positive follicle (%)
80
60
40
20
0
**
*
0
0.001
0.1
0.001
BPA (mg/kg BW)
EB
E
Caspase-3 positive CL (%)
80
60
40
20
0
*
**
*
0
0 001
0.1
0.001
BPA (mg/kg BW)
EB

Figure 2

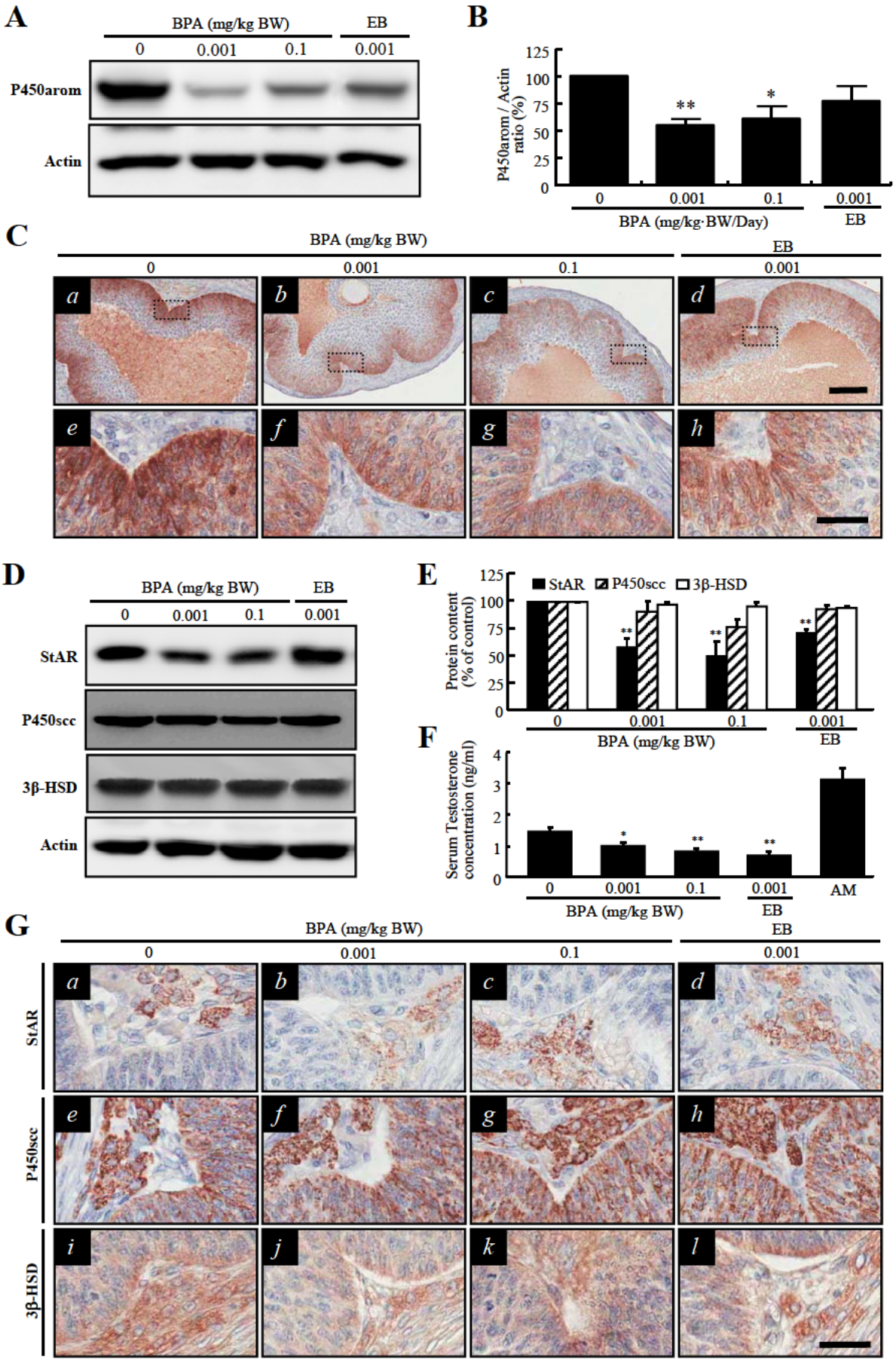
A
BPA (mg/kg BW)
EB
0
0.001
0.1
0.001
P450arom
Actin
B
P450arom / Actin ratio (%)
125
100
75
50
25
0
**
*
0
0.001
0.1
0.001
BPA (mg/kg·BW/Day)
EB
C
BPA (mg/kg BW)
EB
0
0.001
0.1
0.001
a
b
c
d
e
f
g
h
D
BPA (mg/kg BW)
EB
0
0.001
0.1
0.001
StAR
P450scc
3β-HSD
Actin
E
StAR
P450scc
3β-HSD
Protein content (% of control)
125
100
75
50
25
0
**
**
**
0
0.001
0.1
0.001
BPA (mg/kg BW)
EB
F
Serum Testosterone concentration (ng/ml)
4
3
2
1
0
*
**
**
0
0.001
0.1
0.001
AM
BPA (mg/kg BW)
EB
G
BPA (mg/kg BW)
EB
0
0.001
0.1
0.001
StAR
P450scc
3β-HSD
a
b
c
d
e
f
g
h
i
j
k
l

Figure 3

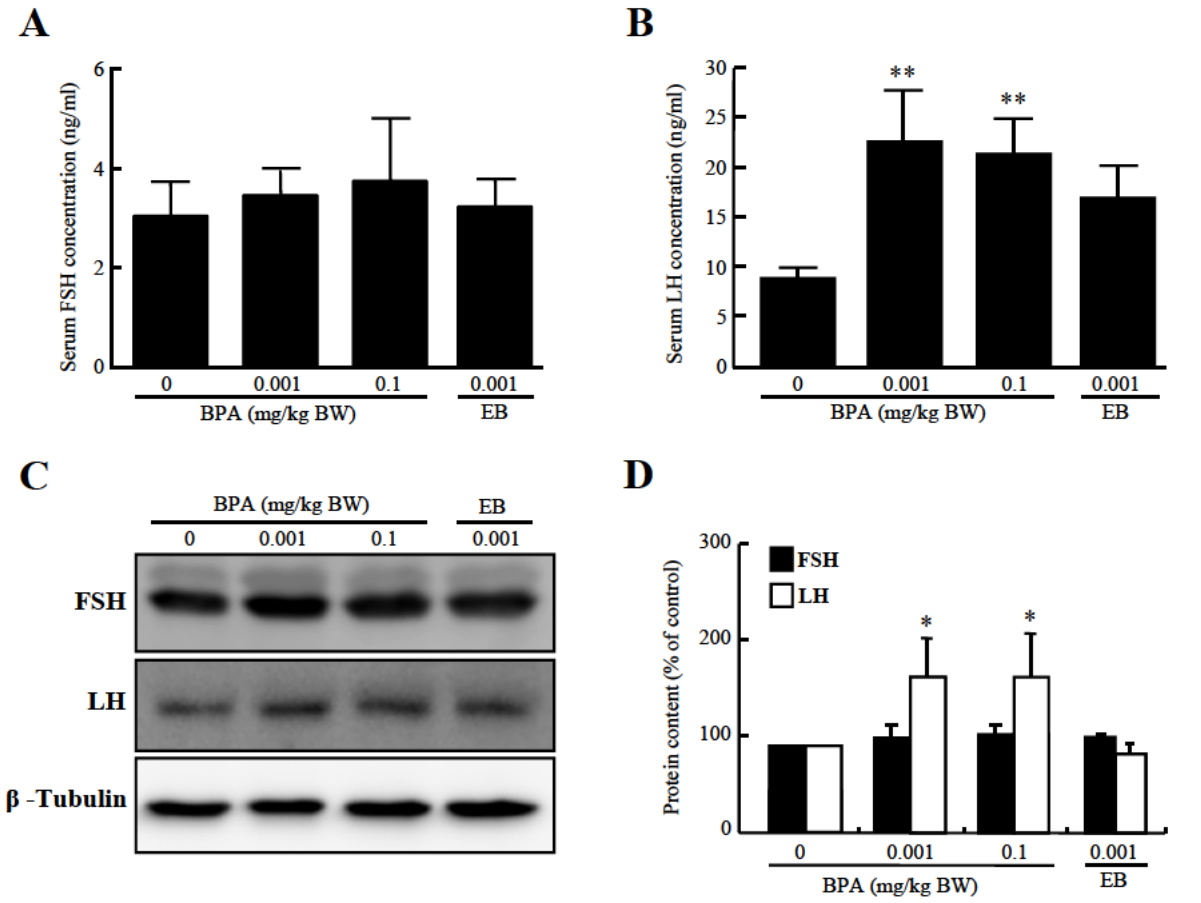
A
Serum FSH concentration (ng/ml)
0
2
4
6
0
0.001
0.1
0.001
BPA (mg/kg BW)
EB
B
Serum LH concentration (ng/ml)
0
5
10
15
20
25
30
**
**
0
0.001
0.1
0.001
BPA (mg/kg BW)
EB
C
BPA (mg/kg BW)
EB
0
0.001
0.1
0.001
FSH
LH
β -Tubulin
D
Protein content (% of control)
0
100
200
300
FSH
LH
*
*
0
0.001
0.1
0.001
BPA (mg/kg BW)
EB
E
BPA (mg/kg BW)
EB
0
0.001
0.1
0.001
FSH
LH
a
b
c
d
e
f
g
h

Figure 4